Cell Differentiation in Human Anagen Hair and Hair Follicles Studied with Anti-Hair Keratin Monoclonal Antibodies

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By a hybridoma technique using BALB/c mice and Sp2/0-Ag14 mouse myeloma cells, monoclonal antibodies against hair fibrous proteins (HFP) were produced. Two monoclonal antibodies, designated as HKN-5 and HKN-7, were chosen. Either HFP or epidermal fibrous proteins (EFP) were electrophoretically separated on polyacrylamide gels with sodium dodecyl sulfate. By immunoblot analyses, HKN-5 and HKN-7 decorated the electrophoretic bands of HFP but not those of EFP. Immunohistochemically, these monoclonal antibodies stained the medulla, cortex, cuticle, and inner root sheath in the keratogenous zone of anagen hairs, but not hair matrix cells. HKN-5 further reacted with the innermost cells (IMC) of the outer root sheath; these cells formed a single cell layer located outside of the Henle's layer. HKN-7 did not react with the outer root sheath including the IMC. Neither monoclonal antibody reacted with any other skin components or any tissues of other organs examined. Ultrastructurally, the IMC of the outer root sheath showed a unique cell differentiation forming an independent cell layer.

It is suggested that the cells in the medulla, cortex, cuticle, and inner root sheath of anagen hair and hair follicles possess a similar keratin expression and that the IMC of the outer root sheath display a unique keratin expression and their own cell differentiation, resulting in 2 types of keratinization of the outer root sheath; keratinization of IMC and trichilemmal keratinization. *J Invest Dermatol 86:563–569, 1986*

Fibrous proteins of keratin are biochemically extracted from various human epithelial cells [1]. Biochemically the hair filaments, one of the main components of hair and a member of the keratin family, are also obtained from hair samples as hair fibrous proteins (HFP) by the established method of Baden et al [2] and their peptide mapping has been clearly demonstrated 1- [2] or 2-dimensionally [3,4] by polyacrylamide gel electrophoresis (PAGE). Several immunologic studies on keratin filaments, using monoclonal antibodies [5–9], have shown that some common antigenic sites of the filaments may be present in different types of epithelial cells, while others seem to be more specific for one type. The epidermal keratinocytes not only change the PAGE pattern of their keratin filament polypeptides during differentiation [2,10], but also display different immunologic staining patterns by monoclonal antibodies in different layers [8,9]. HFP have been immunologically investigated with conventional polyclonal antibodies [2,11–13]; for example, Baden and Kubilus [13] used a polyclonal antibody to HFP for an immunohistochemical survey on skin tissues. In order to detect fine points of hair cell differentiation, more specific monoclonal antibodies seemed to be desirable.

In the present study, 2 monoclonal antibodies were prepared against HFP using extracted HFP from normal human hairs as antigen. Immunohistochemical surveys on human anagen hair and hair follicles and various other human tissues were performed using these 2 antibodies. An ultrastructural study of human hair follicles was also performed to support the immunohistochemical findings.

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Abbreviations:
EFP: epidermal fibrous proteins
ELISA: enzyme-linked immunosorbent assay
HFP: hair fibrous proteins
IMC: innermost cells
2-ME: 2-mercaptoethanol
PAGE: polyacrylamide gel electrophoresis
SCM: S-carboxymethyl
SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Extraction of HFP and Epidermal Fibrous Proteins (EFP) HFP and EFP were extracted from normal human scalp hairs and human callus horny materials, respectively, by the method previously reported [2] and modified.

The hair sample was extracted in 0.2 M Tris/HCl buffer (pH 9.5) containing 8 M urea and 0.2 M 2-mercaptoethanol (2-ME) (Wako Pure Chemical Co., Tokyo) under nitrogen and treated with iodoacetic acid at pH 8.0. After centrifugation, S-carboxymethyl (SCM) low sulfur fraction was precipitated by dialyzing the supernatant against 0.1 M acetate buffer (pH 4.5) containing 0.5 M potassium chloride; this fraction is believed to be HFP [2].

The callus horny material was extracted in 0.1 M Tris/HCl (pH 9.0) containing 8 M urea and 0.1 M 2-ME under nitrogen. After centrifugation, the supernatant was dialyzed against 0.1 M Tris/HCl buffer (pH 7.0); EFP were obtained as the precipitate [2].

HFP are always extracted as an alkylated form, SCM kerateines [14], while EFP can be extracted without such alkylation [15].

Preparation of Hybridomas BALB/c mice were immunized with a mixture of HFP and Freund's complete adjuvant. The spleen cells and mouse myeloma cells (Sp2/0-Ag14) were fused

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using dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Missouri) and polyethylene glycol 4000 (E. Merck, Darmstadt) [16,17]. After washing, these cells were cultured in a medium with fetal calf serum (GIBCO Laboratories), hypoxanthine, aminopterine, thymidine (Sigma Chemical Co.) [18], and peritoneal macrophages derived from nonimmunized BALB/c mice as feeder cells.

Selection of Hybridomas and Production of Monoclonal Antibodies To select hybridomas producing specific antibodies against HFP, hybridoma culture supernatants were tested by an enzyme-linked immunosorbent assay (ELISA) technique [19] using 96-well plates coated with HFP or EFP, β-galactosidase-conjugated antimouse IgG antibody, and *p*-N-β-D-galactopyranoside (BRL, Gaithersburg, Maryland). The hybridomas that produced IgG antibodies reacting with HFP were chosen and cloned 3 times by limiting dilution [20]; thus, each of the hybridomas was producing a monoclonal antibody against HFP. Subsequently, the immunoglobulin subclasses of the monoclonal antibodies were similarly determined by ELISA using rabbit antimouse IgG_1, IgG_{2a}, IgG_{2b}, or IgG_3 antiserum.

Ascites containing high titer of the monoclonal antibody was produced.

Immunohistochemical Study First, cryostat sections 4 μm in thickness of normal human scalp skin materials were examined by using monoclonal antibodies. Two monoclonal antibodies designated as HKN-5 and HKN-7 were chosen and used for further immunohistochemical studies on fresh frozen sections of axillar skin, small intestine, liver, and kidney tissues of human. The frozen section was incubated with a monoclonal antibody (1:10 to 1:1000 diluted ascites) and, then, with fluorescein isothiocyanate-conjugated goat antimouse IgG antibody (Cappel Laboratories, Cochranville, Pennsylvania), mounted in glycerine–phosphate-buffered saline containing paraphenylenediamine (Sigma Chemical Co.) [21] and observed under a Zeiss Standard 18FL fluorescent microscope. After observations, some sections were stained with hematoxylin-eosin or eosin to confirm what structures were fluorescent. Skin sections were also stained with HKN-2, which is one of the anti-hair keratin monoclonal antibodies and has been reported to cross-react with hair tissues and epidermis [22]. As controls, some sections were incubated in a diluted ascites, which was produced with parent myeloma cells, instead of the monoclonal antibody ascites, and similarly processed. The supernatant from the culture of each hybridoma was tested to determine whether it could be used for immunohistochemical survey of skin materials.

Ultrastructural Study Normal human scalp skin specimens with fully developed hair follicles were obtained from 3 individuals. These materials were double-fixed with glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Epon 812 or Araldite. Ultrathin sections of anagen hair follicles were cut with a Sorvall MT-5000 ultramicrotome, double-stained with 1% or 15% uranyl acetate and Reynolds' lead citrate [23], and observed in a JEM 100S electron microscope.

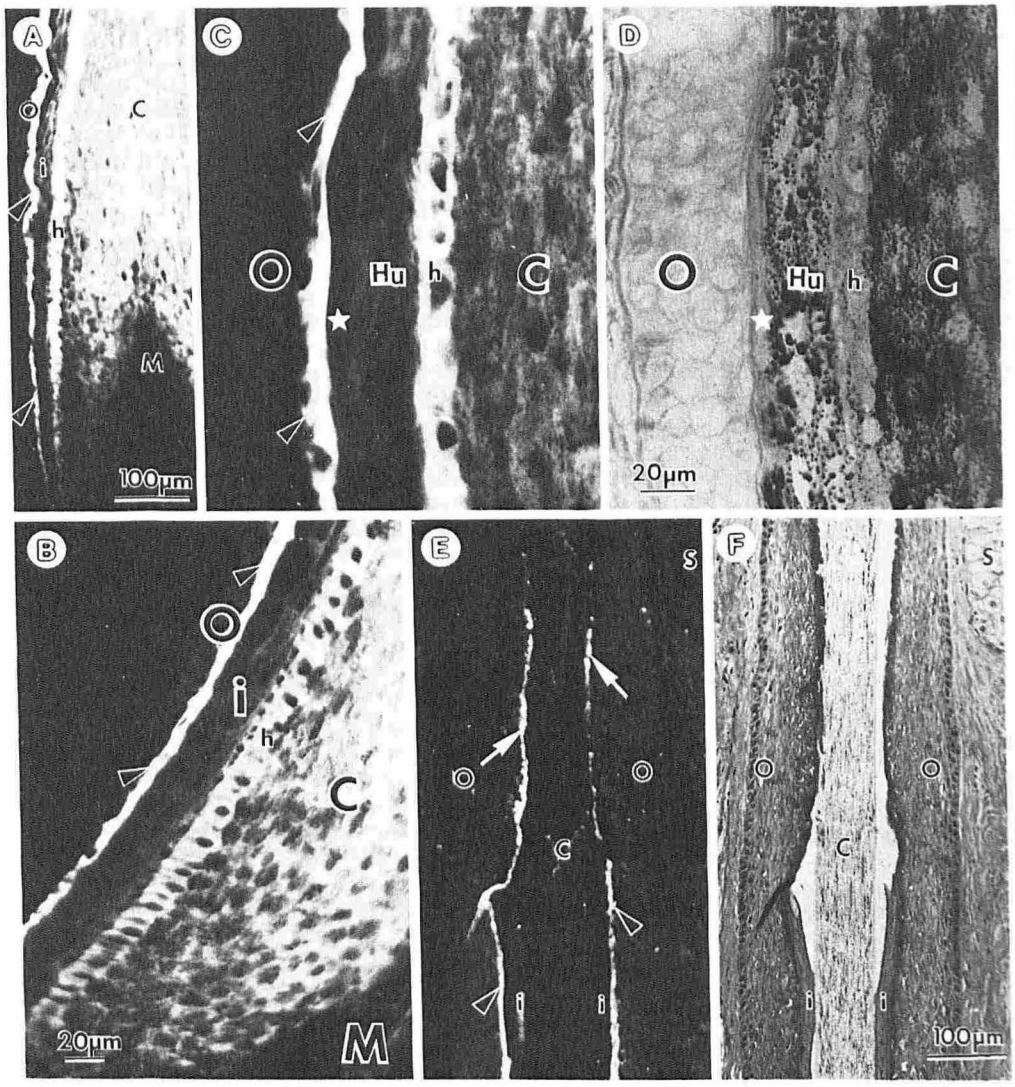

Figure 1. Immunohistochemical reaction of HKN-5 in longitudinal sections of anagen hair and hair follicles of the normal human scalp skin. *A*, Bulbar portion through keratogenous zone. Above the hair matrix (*M*), the immunofluorescence appears in the cells of the cortex (*C*), hair cuticle (*h*), inner root sheath (*i*) and a part (*arrowheads*) of the outer root sheath (*O*), and gradually increases in intensity upward. *B*, Enlargement of a hair bulb. In the outer root sheath (*O*), the IMC (*arrowheads*) show a strong positivity, but other outer root sheath cells no reaction. *C*, Keratogenous zone. *D*, The same section stained with eosin as in (*C*). The IMC (*arrowheads in C*) of the outer root sheath (*O*) located at the outside of the Henle's layer (*star*) show a brilliant fluorescence. *E*, Isthmus portion. *F*, The same section stained with H&E as in (*E*). At the lower part, the IMC (*arrowheads in E*) display a strong fluorescence along outside of the fully keratinized inner root sheath (*i*). This linear fluorescence continues upward and still persists (*arrows in E*) on the surface of the keratinized part of the outer root sheath (*O*) facing the hair canal, although it gradually disappears upward. *Hu*, Huxley's layer; *S*, sebaceous gland. *A*, × 114; *B*, × 300; *C and D*, × 410; *E and F*, × 125.

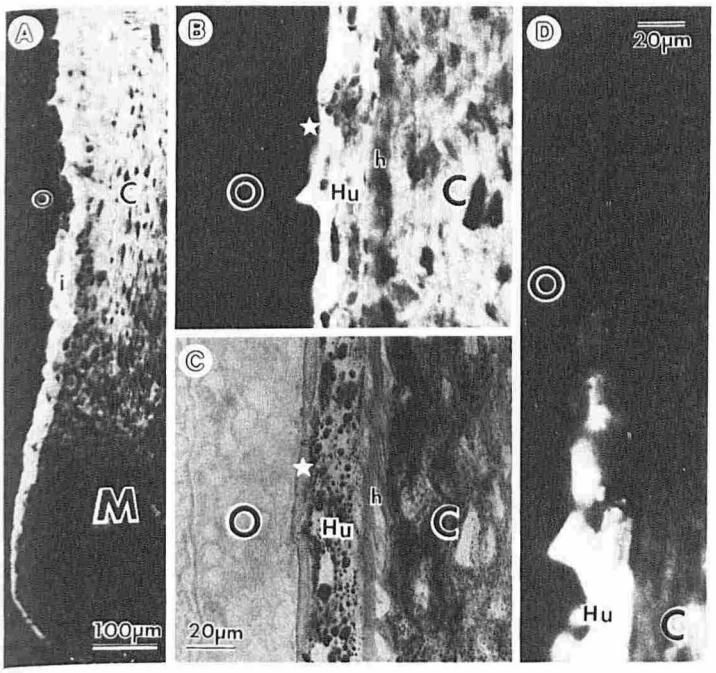

Figure 2. Immunohistochemical reaction of HKN-7 in longitudinal sections of anagen hair and hair follicle in the normal human scalp skin. *A*, Bulbar portion through keratogenous zone. The hair cortex (*C*), hair cuticle, and inner root sheath (*i*) show a strong positivity, but the hair matrix (*M*) and the outer root sheath (*O*) show no fluorescence. *B*, Enlargement of a lower part of keratogenous zone. *C*, The same section stained with eosin as in (*B*). The keratinized Henle's layer (*star*) and the outer root sheath (*O*), including the IMC, show no fluorescence, while other layers (*Hu, h, C*) are strongly stained. *D*, Enlargement of an upper part of keratogenous zone. The Huxley's layer (*Hu*) and hair cortex (*C*) show a fluorescence before keratinization. The keratinized inner root sheath and keratinized hair cortex show no reaction. *h*, hair cuticle. *A*, × 106; *B, C, D,* × 412.

Sodium Dodecyl Sulfate (SDS)-PAGE of HFP and EFP HFP or EFP were dissolved in 50 mM Tris/HCl buffer (pH 6.8) containing 5% 2-ME, 0.5% glycerine, 4 M urea, 0.1% bromphenol blue, and 1% SDS (Eastman Kodak Co., Rochester, New York). Using 10% polyacrylamide (Eastman Kodak Co.) gels (1 mm thick × 60 mm length × 120 mm width) containing 4 M urea, 0.1% SDS and 1% 2-ME, the negatively charged proteins in the HFP or EFP were separated into several bands at pH 8.3 by a current of 20 mA/gel for about 4 h in a vertical slab gel electrophoretic system (Jookoo Co., Tokyo). The standard, Marker I (180K, 140K, 100K, 42K, and 39K; Biochemical Products for Life Science, Tokyo), was simultaneously separated. After electrophoresis, some lanes of the gels were cut, fixed with methanol and acetic acid, and stained with Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, California). The remaining lanes of the gels were used for immunoblot analyses by monoclonal antibodies.

Immunoblot Analyses The electrophoretic bands of HFP or EFP on gels as described above were electrophoretically transferred onto nitrocellulose membrane [24] in Bio-Rad Trans-Blot system (Bio-Rad Laboratories). Each blotted lane on the membrane was separately incubated in a 1:10 to 1:100 diluted ascites containing a monoclonal antibody and reacted with goat anti-mouse IgG antibody (Cappel Laboratories) and, then, with mouse-peroxidase-antiperoxidase (Jackson ImmunoResearch Laboratories, Avondale, Pennsylvania). Finally, the membrane was reacted with 3,3′-diaminobenzidine and hydrogen peroxide.

As controls, some transblotted lanes of HFP or EFP were incubated in a diluted ascites, which was produced with parent myeloma cells, instead of the monoclonal antibody ascites, followed by the same procedure described above.

RESULTS

Immunohistochemical Findings HKN-5 reacted with the inner root sheath, hair cuticle, cortex, and medulla in the keratogenous zone of anagen hairs. The fluorescence appeared in the keratinizing cells of these layers above the hair matrix portion and gradually increased in intensity with their differentiation (Fig 1*A–C*). The reaction of HKN-5 in the outer root sheath was noticed; a strong immunofluorescence was seen in the innermost cells (IMC), which formed a single cell layer in contact with Henle's layer from the bulbar region (Fig 1*B*) through the keratogenous zone (Fig 1*C,D*) to the isthmus portion (Fig 1*E,F*). In the upper part of the isthmus, where so-called trichilemmal keratinization [25,26] begins in many outer root sheath cells, a thin linear or dotted immunofluorescence by HKN-5 remained on the surface of the keratinized outer root sheath; a continuity of this fluorescence with the linear fluorescence of the IMC of the proximal outer root sheath was seen (Fig 1*E*). The intensity of fluorescence decreased toward the opening of the sebaceous duct (Fig 1*E,F*). The cells of the outer root sheath, except for the IMC, displayed no reaction with HKN-5 at any levels (Fig 1*A–C,E*). The hair matrix cells (Fig 1*A,B*) and the keratinized cells in the hair and inner root sheath (Fig 1*C,E*) also showed no reaction.

HKN-7 similarly decorated the inner root sheath, hair cuticle, cortex, and medulla in the keratogenous zone, although the immunofluorescence of the inner root sheath was relatively strong in intensity compared with that of the other layers (Fig 2*A,B*). The outer root sheath including the IMC was never stained with HKN-7 (Fig 2*A,B*). The reaction sites shown in Fig 2*B* are confirmed in Fig 2*C*. HKN-7 did not react with either the hair matrix cells (Fig 2*A*) or the keratinized cells in the inner root sheath (Fig 2*B,D*) and hair shaft (Fig 2*D*).

Both HKN-5 and HKN-7 showed no reaction with any other skin components and other organ tissues examined; the reaction

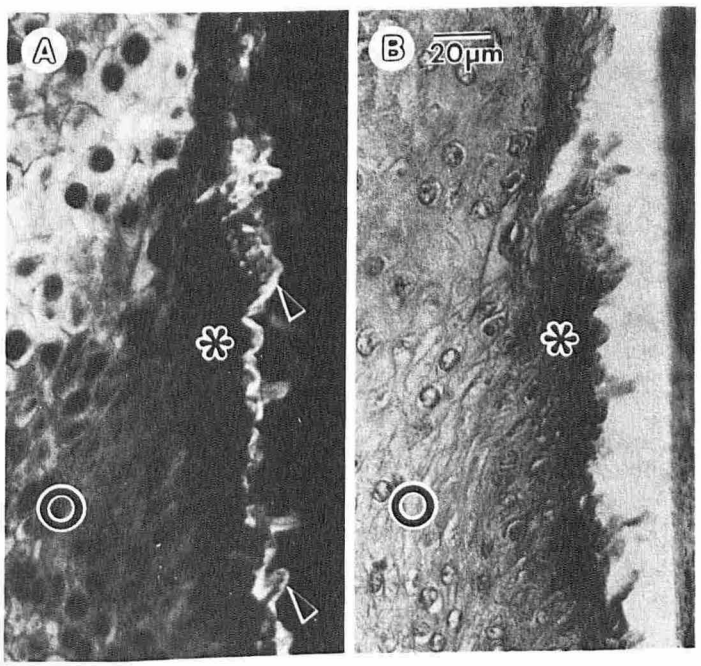

Figure 3. Immunohistochemical reaction of HKN-2 in a longitudinal section of anagen hair and hair follicle of the normal human scalp skin. *A*, Isthmus portion. *B*, The same section stained with hematoxylin and eosin as in (*A*). Positive reaction is seen in the keratinizing cells of the outer root sheath (*O*) but not in the keratinized cells (*asterisks*); while a thin, brilliant immunofluorescence (*arrowheads*) is observed at the surface of the keratinized part facing the hair canal. *A and B,* × 370.

Table I. Reactivities of Anti-Hair Keratin Monoclonal Antibodies with Human HFP and EFP and with Human Tissues

	Anti-Hair Keratin Monoclonal Antibodies		
	HKN-2	HKN-5	HKN-7
Keratin species to recognize:			
HFPs	63K, 72K	72K, 76K	56K, 63K
EFPs	55K, 56.5K	N	N
Tissues			
Pilar apparatus			
Matrix cells	−	−	−
Cells in keratogenous zone[a]	+	+	+
Outer root sheath cells			
IMC	+	+	−
Other outer cells	+	−	−
Keratinized cells	−[b]	−[b]	−
Epidermis, sweat glands and sebaceous glands	+	−	−
Simple epithelia[c]	−	−	−

Key: HFP = hair fibrous proteins
EFP = epidermal fibrous proteins
N = None of the keratin species is decorated by the monoclonal antibody
+ = reacted
− = nonreacted
IMC = innermost cells
[a]Hair medulla, cortex, cuticle, and inner root sheath.
[b]Except for the keratinized IMC of the outer root sheath.
[c]Hepatocytes, bile duct cells, renal convoluted segment cells, parietal cells of Bowman's capsule, intestinal epithelial cells, etc.

sites of these monoclonal antibodies were limited within hair tissues.

HKN-2 reacted with the IMC of the outer root sheath and the cells of the inner root sheath, hair cuticle, cortex, and medulla in a similar pattern to that of HKN-5 as shown above, while other cells of the outer root sheath were also stained with HKN-2. In the portion of trichilemmal keratinization the staining pattern of HKN-2 was of interest; the keratinizing outer root sheath cells showed an intense, but gradually decreasing, immunofluorescence and completely keratinized cells exhibited no positivity with HKN-2, whereas a thin strong fluorescence was observed along the surface of the keratinized outer root sheath facing the hair canal (Fig 3*A*). This linear fluorescence corresponded well to the pattern shown by HKN-5 at the same level (compare Fig 1*E* with Fig 3*A*). The reaction sites shown in Fig 3*A* are confirmed in Fig 3*B*.

All reactions observed above by the 3 monoclonal antibodies were localized in the cytoplasm of epithelial cells and not in their nuclei. None of the monoclonal antibodies reacted with either mesenchymal or neural tissues, including fibroblasts, histiocytes, blood cells, vascular structures, pilar muscles, collagen and elastic fibrils, nerve structures, etc. Control sections showed no positive fluorescence. The farther diluted ascites was used, the weaker the intensity of the immunofluorescence gained, especially in the hair cortical cells in the keratogenous zone. The skin sections stained

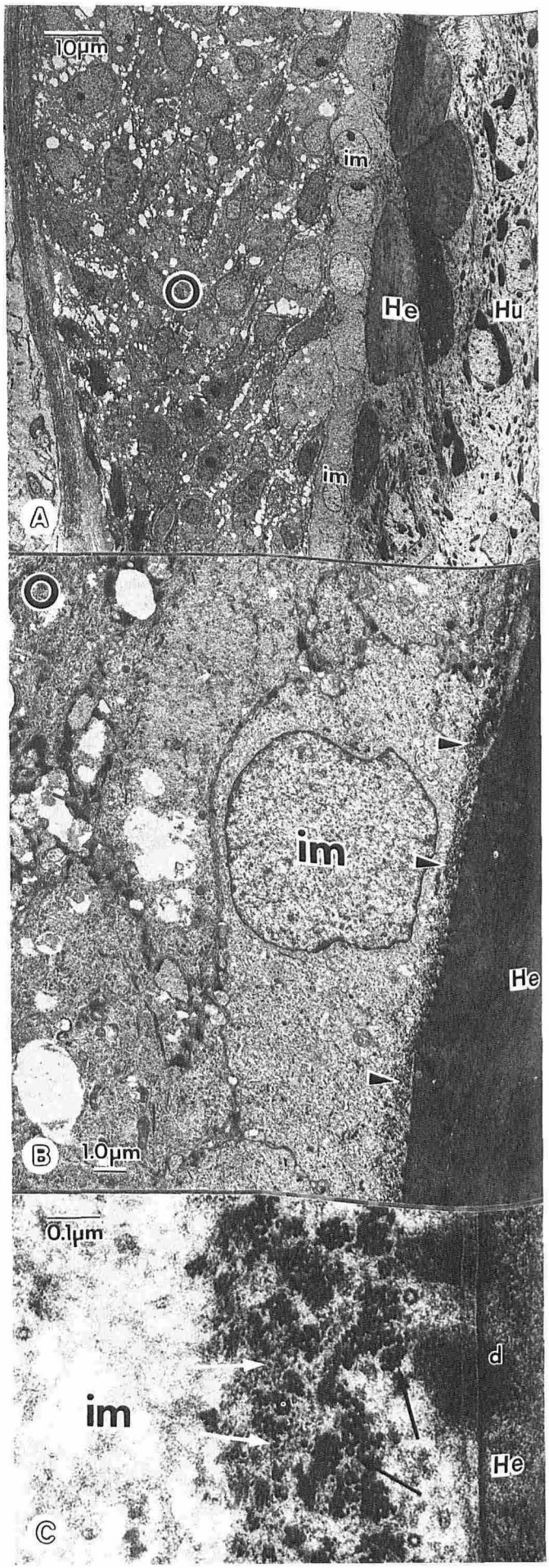

Figure 4. Ultrastructures of longitudinally sectioned anagen hair follicle of the normal human scalp skin. *A*, Suprabulbar portion. The IMC (*im* of the outer root sheath show a linear arrangement along the outside of the keratinizing Henle's layer (*He*); while other cells of the outer root sheath (*O*) show an irregular arrangement. *B*, IMC of the outer root sheath. Tonofilaments (*arrowheads*) are accumulated in the inner side of the cytoplasm of the IMC (*im*). *C*, Enlargement of the accumulated tonofilaments in the IMC. Only cross sections of tonofilaments are seen and show 2 types of ultrastructural images: an electron-dense circle with a lucent core (*black arrows*) and an electron-lucent circle with a dense core (*white arrows*). Uranyl acetate-lead citrate staining. *d*, desmosome. *A*, × 930; *B*, × 5,600; *C*, × 84,000.

by the supernatant from the culture of each hybridoma showed the same results described above.

The reaction sites of the anti-hair keratin monoclonal antibodies in human tissues are summarized in Table I.

Ultrastructural Findings Since the IMC of the outer root sheath were shown to possess a unique keratin differentiation as detected by HKN-5 and HKN-2, the IMC were ultrastructurally observed. Careful attention was paid to the relationship between the outer and inner root sheaths. The anagen hair follicle was always sectioned longitudinally in a plane containing its hair axis.

In the bulbar portion, one layer of flattened cells composed the inner part of the outer root sheath and covered the differentiating Henle's cells inside. At the level between the suprabulbar portion and the keratinizing zone of the Henle's cells, where the Huxley's cells were not yet keratinized and had large trichohyaline granules, the number of cells in the outer root sheath was gradually increasing upward; the IMC of the outer root sheath were still flattened and well arranged parallel to the keratinizing Henle's layer (Fig 4*A*). The IMC were oblong in shape and tonofilaments were accumulated in the inner side of their cytoplasm facing the Henle's cells (Fig 4*B*). These tonofilaments grouped and always ran transversely against the hair axis so that their cross sections were revealed in a longitudinal section of a hair follicle (Fig 4*C*). The cross sections of these tonofilaments showed 2 types of ultrastructural images; an electron-dense circle with a lucent core, or an electron-lucent circle with a dense core. Both types of tonofilaments possessed almost the same diameter of 8–12 nm and gathered in groups, thus producing a patchy appearance of electron-dense and electron-lucent areas (Fig 4*C*). Such distribution of tonofilaments was not found in the other outer cells. The IMC were closely attached to the Henle's cells with desmosomes (Fig 4*C*).

At the level where both Henle's and Huxley's layers were fully keratinized, the IMC were oblong to cuboid in shape, contained an increased number of tonofilaments, and often produced a small number of keratohyaline granules in the cytoplasm. In the isthmus of hair follicles, the IMC displayed a further cell differentiation. At the level where the keratinized cells in the Huxley's layer underwent a degeneration process but those in the Henle's layer still preserved their cell structure and electron density, the IMC became flattened again and showed keratinization (Fig 5). At this level other outer root sheath cells were also flattened in a longitudinal direction along the hair axis but never keratinized (Fig 5). More distally, where so-called trichilemmal keratinization occurred [25,26], the IMC still had contact with the Henle's cells and were fully keratinized; however, there was no significant difference in ultrastructure between the keratinized IMC and other keratinized cells of the outer root sheath.

At the upper part of the isthmus just below the opening of the sebaceous duct, where no structures of the inner root sheath were seen, a degeneration of the keratinized cells of the outer root sheath facing the hair canal occurred and a morphologic identification of the IMC became impossible.

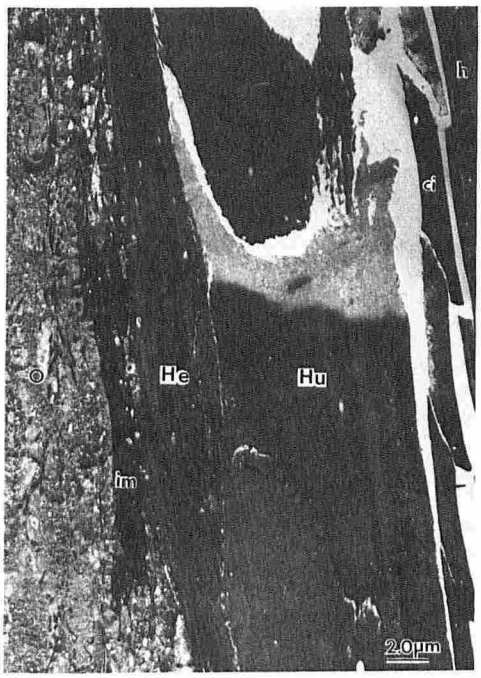

Figure 5. Keratinization of the IMC. At the level where the keratinized Huxley's cells (*Hu*) undergo a degeneration but the Henle's cells (*He*) still preserve their keratinized cell structures, the IMC (*im*) shows a lamellar shape and keratinization. The other cells of the outer root sheath (*O*) exhibit no sign of keratinization. Longitudinal section of anagen hair follicle stained with uranyl acetate-lead citrate. *ci*, cuticle of the inner root sheath; *h*, hair cuticle, × 4,050.

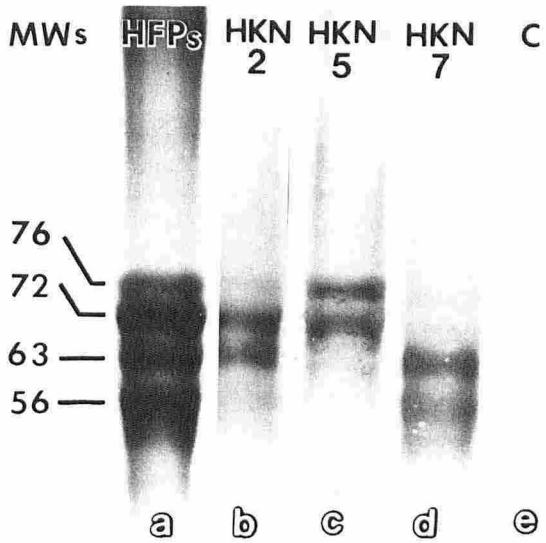

Figure 6. SDS-PAGE of HFP and immunoblot analysis with anti-hair keratin monoclonal antibodies. HFP were separated on 10% polyacrylamide gel with SDS and stained with Coomassie Brilliant Blue. Four major bands of *M*, 76K, 72K, 63K, and 56K are seen (*lane a*). By immunoblot analysis, Each of the monoclonal antibodies, HKN-2 (*lane b*), HKN-5 (*lane c*), and HKN-7 (*lane d*), shows a specific reaction with the bands of HFP in a unique pattern. C, control (*lane e*).

Immunoglobulin Subclass of Anti-Hair Keratin Monoclonal Antibodies The monoclonal antibodies, HKN-5 and HKN-7, were in a subclass of mouse IgG_1 by ELISA.

Reactivity of Monoclonal Antibodies Against HFP or EFP by ELISA By ELISA, HKN-2 reacted with either HFP or EFP, while HKN-5 and HKN-7 showed a reactivity only with HFP.

Findings of SDS-PAGE and Immunoblot Analyses By SDS-PAGE, HFP and human callus EFP were separated into 4 major bands of *M*, 76K, 72K, 63K, and 56K (Fig 6) and 5 major bands of *M*, 67K, 66K, 58K, 56.5K, and 50K (Fig 7), respectively.

Immunoblot analyses showed that HKN-2 specifically reacted with 2 major bands of 72K and 63K HFP (Fig 6), while 1 major band of 56.5K and 1 minor band of 55K EFP were decorated by HKN-2 (Fig 7) [22]. Although HKN-5 revealed a reactivity with 2 major bands of 76K and 72K HFP (Fig 6), none of the migrated bands of EFP was decorated by HKN-5 (Fig 7). Similarly, HKN-7 reacted with 2 major bands of 63K and 56K of HFP (Fig 6) but not with the bands of EFP (Fig 7). Control lanes of either HFP

(Fig 6) or EFP (Fig 7), treated with ascites produced by parent myeloma cells, showed no positive staining. The M_r sizes of the reacted bands of HFP and EFP are listed in Table I.

DISCUSSION

Weber and Osborn [12] showed that the polyclonal antibodies against wool-merokeratin, modified HFP, stained nonkeratinizing epithelial cells. Recently, Baden and Kubilus [13] performed an immunohistochemical survey on hard keratin-producing tissues including hair, using polyclonal antibodies against HFP, and found that the antibody reacted with the developing cells in the hair cortex, but not with the cells in the epidermis and outer root sheath. As shown in the present study, however, the preparation of a monoclonal antibody seems to allow us to examine the localization of an antigenic determinant in extracted proteins or in tissue sections with an extremely high titer of the antibody; more precise immunologic characteristics of proteins seem to be understood by using monoclonal antibodies than by polyclonal ones.

Previous investigators produced monoclonal antibodies against EFP and discussed the production and differentiation of polypeptides of EFP in the epithelial cells during cell differentiation [8,9]. Monoclonal antibodies against wool fibrous proteins were also produced by other investigators [27]. However, hair tissues have not been immunohistochemically examined with these monoclonal antibodies. The inner root sheath, cuticle, cortex, and medulla in the keratogenous zone of hair tissue were similarly decorated by both HKN-5 and HKN-7 (see *Results*) and also by HKN-2 [22], indicating that the cells in these layers display a similar keratin expression. On the other hand, the reactivities of the outer root sheath with the monoclonal antibodies are quite different. Especially, as shown by HKN-5, the IMC of the outer root sheath have a unique keratin component which is not present in other cells of the outer root sheath. The IMC are also immunologically distinguished from the inner root sheath cells in reactivity with HKN-7.

Ultrastructurally, a few investigators [26,28,29] have examined the outer root sheath; however, little attention has been given to the IMC and no sequential examination of the ultrastructures of these cells from the hair bulb to keratogenous zone has been performed. From the present ultrastructural observation, the IMC are considered to form a unique and independent layer, which is easily distinguished from other cells of the outer root sheath (see *Results*). Pinkus et al, who proposed the term *trichilemmal keratinization* for the keratinization of the outer root sheath at the isthmus [25] and described its ultrastructure [26], stated that the IMC were flattened, produced no keratohyaline granules, and did not become keratinized until the keratinized Henle's cells sloughed. However, this concept should be modified and changed by the present ultrastructural findings. As shown by HKN-5 and HKN-2, the immunologic feature of the IMC is persistent until they become completely disintegrated. It is concluded that the outer root sheath of human anagen hair follicle undergoes 2 types of keratinization; keratinization of IMC and so-called trichilemmal keratinization.

The immunologic reactivities of HKN-2, HKN-5, and HKN-7 to HFP by immunoblot analyses reveal that these monoclonal antibodies certainly recognize antigenic determinants present in the keratin components in keratinized hairs. Immunohistochemically, however, the keratinized hair structures are not decorated by these monoclonal antibodies. In the extraction procedure of HFP from hairs, the keratin filaments in the keratinized hairs became fragmented into small components by 2-ME and iodoacetic acid; this fragmentation might open up the intrinsic antigenic sites in the fibrous proteins. The monoclonal antibodies probably react with such antigenic sites, which may be masked by a structural modification of the molecules of the keratin filaments during keratinization of hair. Indeed, the developing hair cells in the keratogenous zone are immunohistochemically decorated by these monoclonal antibodies without any pretreatments of frozen skin sections before the staining procedure. The immunologic differences of fibrous proteins in the developing cells among the hair medulla, cortex, cuticle, and inner root sheath are unknown and are still under investigation.

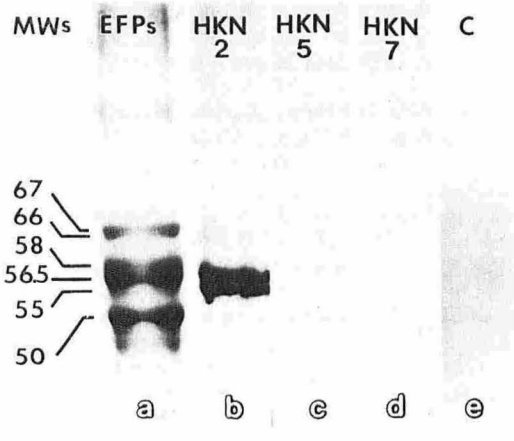

Figure 7. SDS-PAGE of EFP and immunoblot analysis with anti-hair keratin monoclonal antibodies. EFP were separated on 10% polyacrylamide gel with SDS and stained with Coomassie Brilliant Blue. Five major bands of M_r 67K, 66K, 58K, 56.5K, and 50K and some minor bands are seen (*lane a*). By immunoblot analysis, HKN-2 (*lane b*) specifically reacts with the bands of EFPs; however, HKN-5 (*lane c*) and HKN-7 (*lane d*) show no reaction. C, control (*lane e*).

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